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PRINCIPAL INVESTIGATOR: Michael Aschner, Ph.D.

CONTRACTING ORGANIZATION: Wake Forest University School of
                          Medicine
                          Winston-Salem, North Carolina 27157

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**INTRODUCTION (Based on original abstract)**

Recent studies of Gulf War veterans with depleted uranium (DU) embedded fragments in their soft tissues point to DU-induced effects on neurobehavioral and cognitive function (McDiarmid et al., 2000). These observations are corroborated by electrophysiological changes in hippocampal slices isolated from rats embedded with DU fragments (Pellmar et al., 1999a; Pellmar et al., 1999b). Notably, studies from the same group also suggest, for the first time, that uranium accumulates within brain tissue (Pellmar et al., 1999a). It is presently unknown how uranium is transported into the brain, and there are no pharmacological modalities to reduce its accumulation within the central nervous system (CNS). The purpose of this project is to identify the substrate specificity of uranium transport in the CNS, the working hypothesis being that the divalent metal transporter (DMT-1) which has an unusually broad substrate range that includes $Fe^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Ni^{2+}$, and $Pb^{2+}$, is mediating uranium transport in the CNS. This project focuses on examining this hypothesis from an in vitro approach utilizing endothelial cell culture models (Technical Objective 1.0) as well as an in vivo approach to delineate the pharmacokinetics of uranium transport across the BBB in rats embedded with DU fragments (Technical Objective 2.0). The studies will test the hypothesis that a relationship exists between blood and brain uranium concentrations, determining whether rats with the highest blood uranium concentrations also accumulate the highest uranium concentrations in the CNS. Thus, the studies will facilitate risk assessment in veterans, and will determine whether those with the highest uranium blood levels are more prone to accumulate uranium in the CNS compared to veterans with low blood uranium levels.

**BODY**

Stated Technical Objectives for Year 2 of this proposal were:

1.0  To determine the in vitro transport of uranium across the BBB in *in vitro* endothelial cell culture models (RBE4 and bovine brain endothelial cells).

2.0  Study the *in vivo* transport of uranium across the BBB.


**SUMMARY REVIEW:**

**Technical Objective 1:**

The purpose of this study is to assess the substrate specificity of uranium (U) transport into the central nervous system.  The Principal Investigator (PI) plans to examine the in vitro transport of U across the blood-brain barrier (BBB) in endothelial cells in vitro and to study the in vivo transport of U across the BBB in a rat model.

During the third year of the study the PI cultured rat brain endothelial 4 cells (RBE4) for in vitro assessment of U transport and end points of neurotoxicity. The following sections summarize the results that have been obtained during year 3 of the proposal.

**Volume measurements in RBE4 cells treated with U**

RBE4 cell volume was determined by modification of the electrical impedance method. Cell monolayers grown on a #1.5 coverslip were placed in a channel, which joins two chambers. The total height of the channel was approximately 250 µm. Each chamber contained a silver wire electrode (5 cm length) that was soldered at the distal end to a copper insulated wire for connection through a large resistance (1 megohm), to a lock-in amplifier that supplied a 500 Hz, 5 volt signal to the system. There was a continuous flow of solution through the channel (1 ml/min). As the volume of the cell monolayer increased, the volume of the solution within the channel and above the cells available for current flow decreases proportionately, resulting in an increase in the measured resistance in the channel above the cells. Because $V=IR$ (where V is voltage, I is current and R is resistance) and I was kept constant, changes in V are directly proportional to changes in R.

The monolayer of RBE4 cells was bathed in a HEPES-buffered solution consisting of the following: 122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 10 mM D (+) glucose, 25 mM HEPES. All buffers were adjusted to pH 7.4 by addition of 10 N NaOH. The osmolarity of the isotonic solution was approximately 300 mOsmol as measured by a freezing point

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osmometer (Advanced Instruments, Inc., Needham Heights, MA.).
The protocol for all experiments involved a 15 min exposure to
isotonic buffer, followed by a 60 min exposure to isotonic
buffer containing various concentrations of $U_3O_8$ (0-100 uM).

Volume changes (Figure 1) are expressed as change in voltage.
As can be noted in Figure 1, the maximal change in voltage was
an increase from approximately 140 mV for controls to 140.75
mV in RBE4 cells treated with 100 uM $U_3O_8$.  This translates to
about 0.5% change in cell volume. Since a one percent change
in volume in our system approximates a 25% change in cell
volume (assuming cellular cell height of 4 uM) we conclude
from these experiments that $U_3O_8$ does not cause a drastic
change in cell volume over the 30-minute exposure period.
Thus, RBE4 cells are capable of withstanding a high exposure
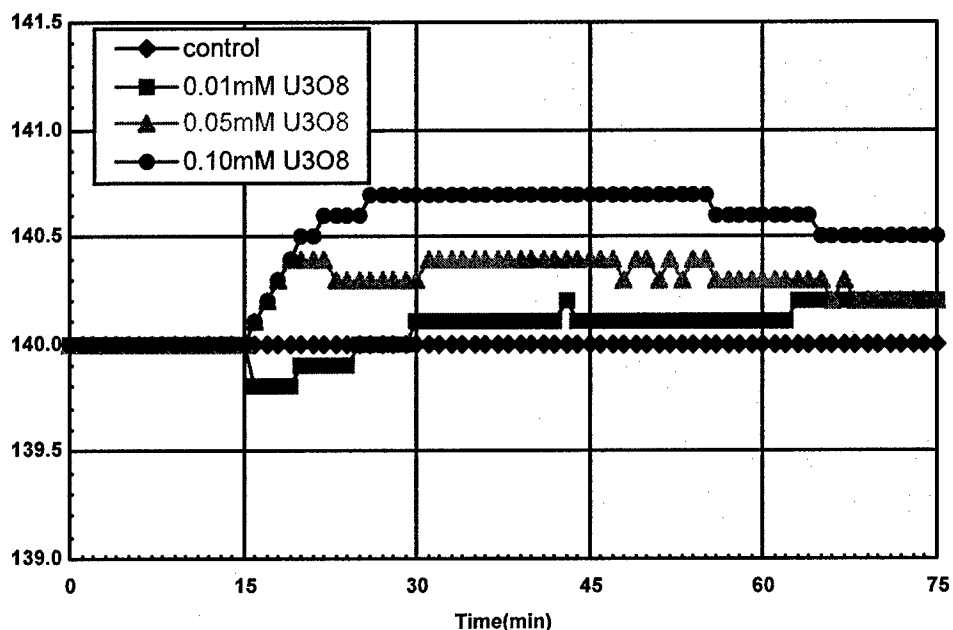to U without a discernable change in cellular volume.



Figure 1

**Effects of U on Hsp90 expression in RBE4 cells**
The 90-kDa heat shock protein (Hsp90) is one of the most
abundant proteins in cells, constituting 1-2% of total
intracellular protein. It is constitutively and ubiquitously
expressed and is the most abundant molecular chaperone of the

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eukaryotic cytoplasm. Chaperones help to achieve and maintain the conformational status of cellular proteins and enzyme complexes. By influencing higher order protein structure, Hsp90 is involved in the conformational regulation of key proteins in multiple signaling pathways, including nitric oxide synthases (NOS), kinases, phosphatases and steroid hormone receptors. Geldanamycin, an ansamycin antibiotic that binds to the ATP binding site of Hsp90, and the structurally unrelated macrocyclic antifungal, radicicol, both disrupt the association of Hsp90 with its client proteins, and have been useful in elucidating the functional roles of Hsp90 in cell signaling. Geldanamycin and radicicol bind with a higher affinity to the same site on Hsp90 as ATP, suggesting that protein folding is an ATP-dependent process. It is important to note, however, that inhibition of Hsp90-mediated protein folding by geldanamycin and radicicol *does not* prevent binding of Hsp90 to client proteins or disrupt existing Hsp90/client protein complexes.

Hsp90 regulates the redox status of other proteins by assisting in the formation and breakage of disulfide bridges. Reactive cysteines are usually found in the vicinity of the ATP binding site of chaperone proteins. The cysteine groups participate in the binding of Hsp90 with its client proteins and with molybdate, a stabilizer of Hsp90-client protein interactions. The high redox reactivity of its cysteines (cysteines 521 and 589/590) provides the mechanism by which this molecular chaperone helps maintain the redox status of the cytosol. Oxidizing conditions impair the chaperone activity of Hsp90, further proof of the active participation of sulfhydryl groups in the function of Hsp90. Furthermore, normal coupling between Hsp90 and nNOS is important for normal nNOS function. Disruption of this coupling may result in superoxide ($\cdot O_2^-$) overproduction, which could in turn lead to neuronal nitric oxide (nNOS) dysfunction and CNS pathology. The importance of Hsp90/nNOS interactions in the promulgation of neurotoxicity is incompletely understood. Furthermore, association of nNOS with Hsp90 opens the thiol moieties in the ligand-binding domain to attack by thiol-derivatizing agents. The potential for U-mediated disruption of Hsp90/NOS interactions is totally unexplored. As a first step in this process we have carried out studies using routine western blot techniques to assess the ability of $U_3O_8$ to alter Hsp90 expression in RBE4 cells. As shown in Figure 2, $U_3O_8$ caused a concentration-dependent significant ($p<0.05$) increase in Hsp90 levels at 10 and 30 minutes (100 and 10 uM, respectively) compared to control RBE4 cells, but this effect was not apparent after 1 hour (note y-axis represents % change over

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control, which is standardized to 100%). Accordingly, we conclude that this effect of $U_3O_8$ is fast and short lasting, yet the consequences will have to be probed at further detail in futures studies.
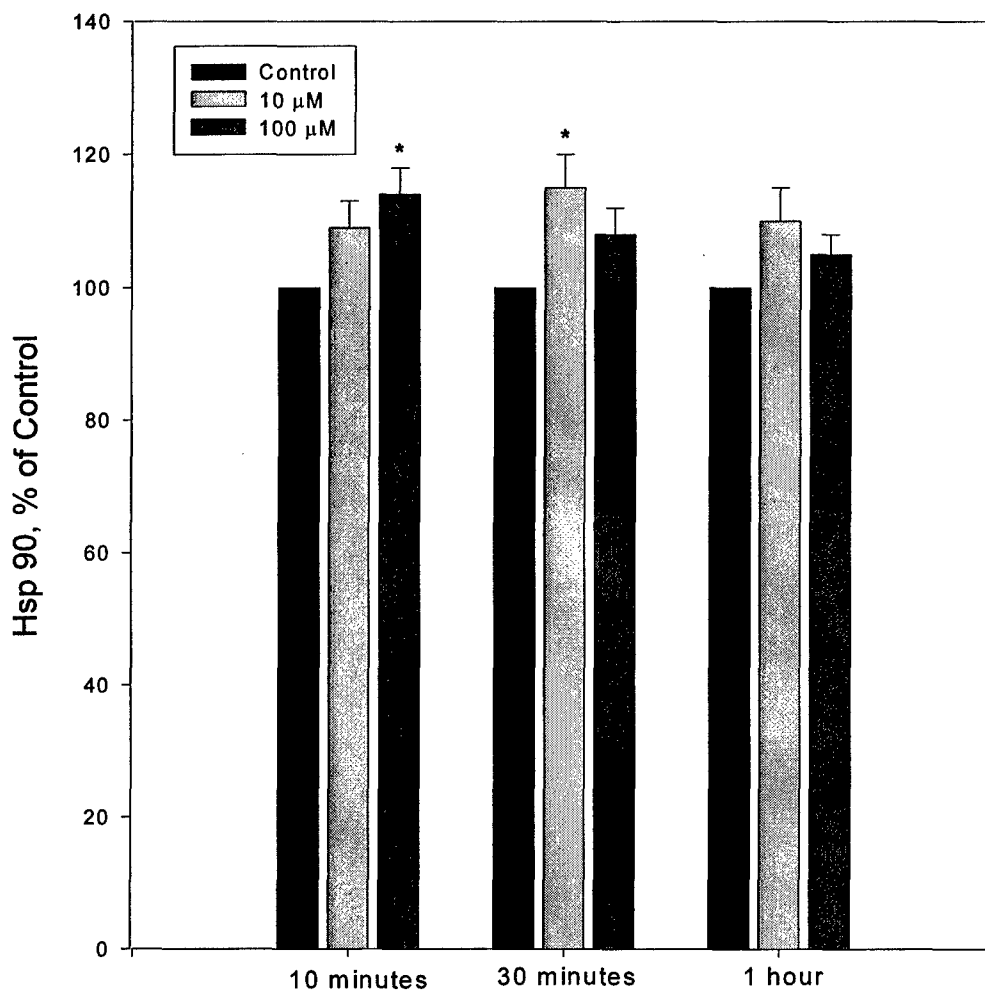


Figure 2

**Technical Objective 2:**
Male Sprague-Dawley rats, initial mass of ~250g, were ordered from Harlan. Rats were randomly divided into two cohorts, a 3-month or 6-month cohort. Five different groups were randomly assigned within each of the cohorts: non-surgical controls (NS Control) that did not receive any treatment other than weekly handling for weighing, sham animals that received 10 tantalum (Ta) and zero depleted uranium (DU) pellets per leg, low dose animals that received 2 Ta and 8 DU pellets per leg, medium

dose animals that received 5 Ta and 5 DU pellets per leg, and high dose animals that received 10 DU and zero Ta pellets per leg. Rats were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine, and surgery did not begin until animals were in a deep phase of anesthesia according to their response to a toe pinch. Using sterile surgical techniques, a 2-3 cm incision was made in the gastrocnemius on both legs (see pages 10 and 11 for figures). A total of 10 pellets were placed in the muscle on each side (Figure 3). Incisions were stitched up with suture and animals were allowed to recover in a warm insulated box until fully conscious. Animals were monitored daily for three days to insure that no infection occurred. No animals in the study were lost following surgery due to infection. Post-surgical survival rate was 100%.

Because control animals for the 3-month cohort were not weighed for the first three weeks, the data is presented as 'Weight gain (%)' from the first week and from the third week, which is more accurate (Figure 3 and 4, respectively). Although overall, there was no significant weight gain by one-way ANOVA, it appears that the DU animals did not differ in weight gain vs. controls regardless of how the data is standardized (weight gain from week one or week three).

**Percent Change in Weight Gain
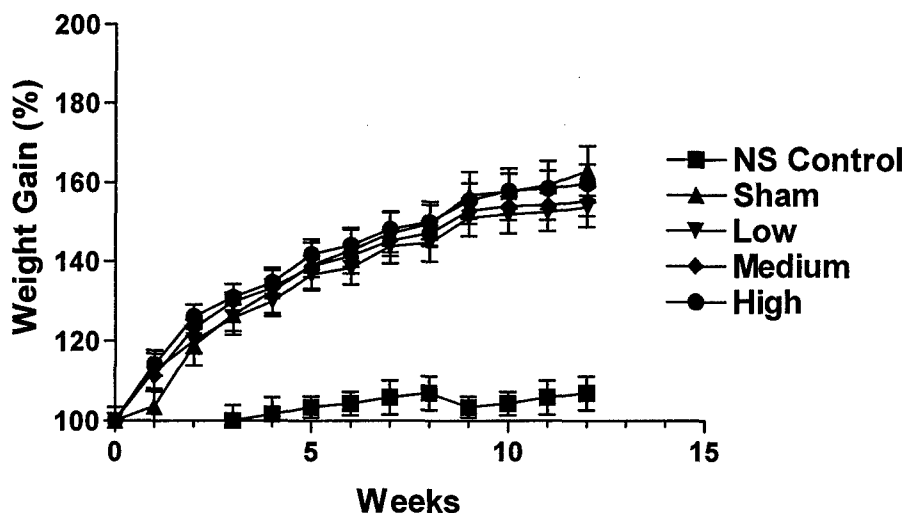for Depleted Uranium-Treated
Rats: 3-Month Group**



Figure 3

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**Percent Change in Weight Gain
for Depleted Uranium-Treated
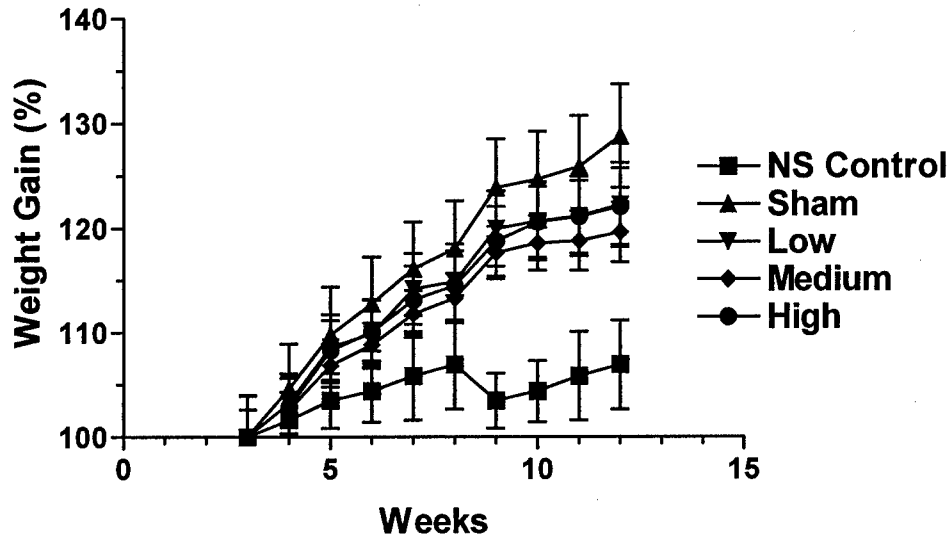Rats: 3-Month Group**



Figure 4

**Percent Change in Weight Gain
for Depleted Uranium-Treated
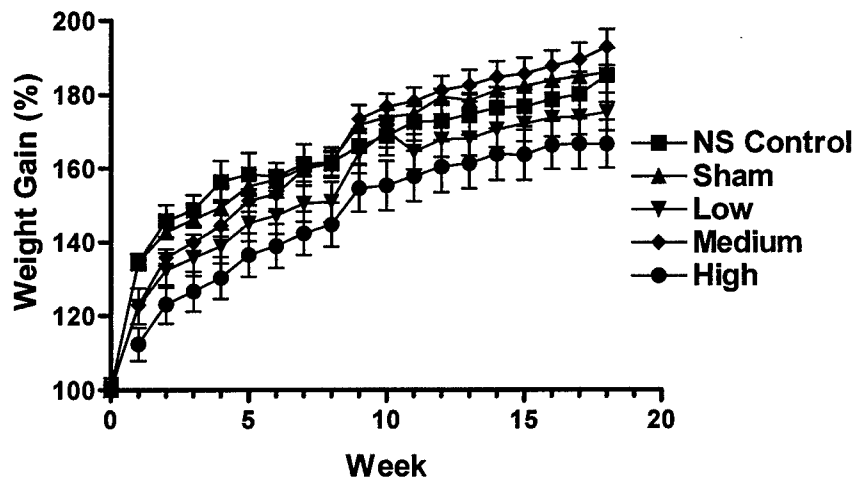Rats: 6-Month Group**



Figure 5

The weight gain for the 6-month rat cohort was determined for the whole duration as shown in Figures 5 and 6. There were no significant weight gain differences among any of the groups in the 6-month cohort whether expressed as weight gain (%) (Figure 5) or total weight gain (Figure 6).

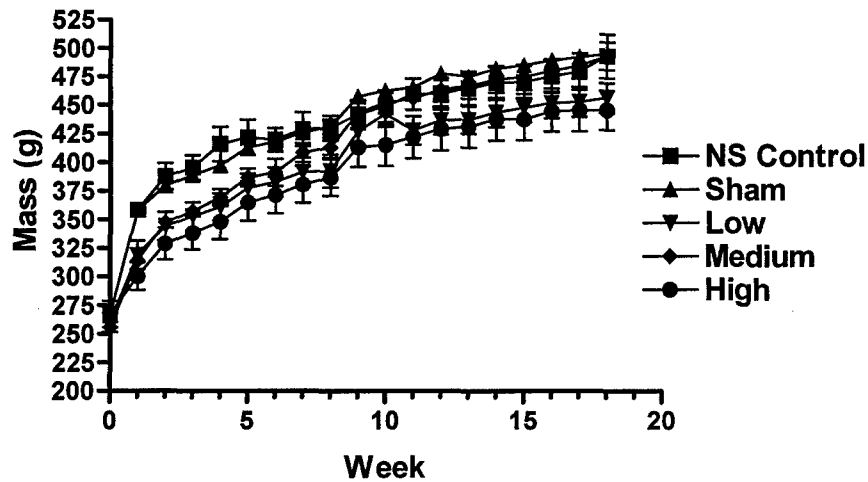**Average Group Weight Gain for Depleted Uranium-Treated Rats: 6-Month Group**



Figure 6

Figure 7 shows DU pellets on a metric scale. Figure 8 shows the implanted DU pellets in the rat gastrocnemius muscle.
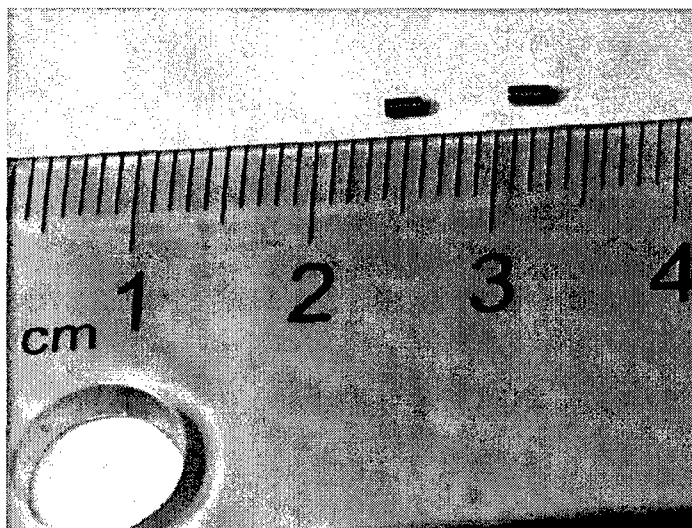
Figure 7



Figure 8


**Measurements of Brain U**

Animals were sacrificed at 3 months or 6 months, depending on
group designation. Animals were initially anesthetized again
with 80 mg/kg ketamine, 12 mg/kg xylazine prior to perfusion
with a total volume of 200 mL phosphate buffered saline (PBS),
pH 7.4. Following perfusion, animals were decapitated and
brains were dissected into the following groups: cerebellum,
midbrain, brain stem, striatum, hippocampus and cortex. All
section were wrapped in aluminum foil and quick-frozen by
placing the sections in dry ice. Major organs, although not
reported here, were also removed and they have been frozen at
-80°C should we wish to carry out any analysis in the future.

To date, only hippocampus and cortex from three animals in the
6-month cohort have been analyzed for U content using
inductive capillary plasma - mass spectroscopy (ICP-MS). We
opted to start the analysis with the 6-month cohort given that
we felt that it would be the cohort likely to show the most U
accumulation (reflecting upon the longest exposure period).
Our collaborator at Trondheim University, Trondheim Norway,
has developed the necessary technique and quality control. The
rationale for our choosing of hippocampus and cortex is
because the 2 CNS areas have been classified as target and
non-target areas for U, respectively (Pellmar et al., 1999b).

HR-ICP-MS analysis was performed using a Thermo (Finnigan) model Element instrument (Bremen, Germany). The RF power was 1150 W. The sample was introduced using a CETAC ASX 500 autosampler (Omaha, USA) with a peristaltic pump (pump speed 1 mL per min). The instrument is equipped with a concentric Meinhard nebulizer connected to a Scott spray chamber, and a quartz burner with a guard electrode. The nebulizer argon gas flow rate was adjusted daily to give a stable signal with maximum intensity for the nuclide $^{115}$In.

The instrument was calibrated using 0.5 M $HNO_3$ solutions of multielement standards at appropriate concentrations. Internal standards were not used. After each sample 0.1 M $HNO_3$ (Suprapur) was flushed through the sample introduction system to reduce memory effects. To check for possible drift in the instrument, a standard solution with known elemental concentrations was analyzed for every 10 samples. In addition, blank samples (0.5 M $HNO_3$, Suprapur) were analyzed for approximately every 10 samples. The samples were analyzed in random order, and the analyst did not know the identification of the samples. U concentrations were determined in the medium resolution mode ($M/\Delta m=4000$). The relative standard deviation was generally 10% or better. The reagent blanks were nearly constant and negligible (<1%).

As shown in Figure 9, one-way ANOVA indicates that there is no statistically significant difference in the amount of U in any of the brain regions for the various DU treatments. It is noteworthy that ours are the first studies in which whole animal perfusions have been carried out prior to sacrifice (in contrast for example to Pellmar et al., 1999a). We are most excited and confident in this data given that our results for the U brain concentrations in controls are identical to those reported by Pellmar et al. (1999a), in which a different method was used. We feel strongly that the measured U concentrations reflect accurate brain levels, and we believe that the reason they contradict results reported by others is that in our case contamination of the samples by blood-borne U was minimal, whilst in other studies (Pellmar et al., 1999a) contamination by U from blood may not be ruled out. Though at this point our studies are not definitive and we are in the process of analyzing shorter exposure cohorts, we believe that to date the data suggest that U may not readily enter the CNS.
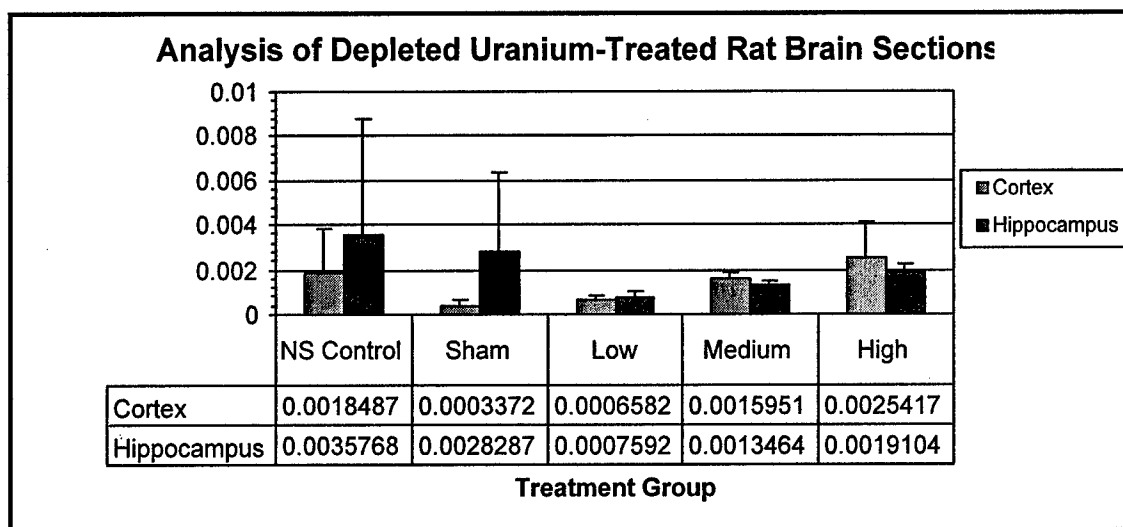
**Analysis of Depleted Uranium-Treated Rat Brain Sections**

| Treatment Group | NS Control | Sham | Low | Medium | High |
|---|---|---|---|---|---|
| Cortex | 0.0018487 | 0.0003372 | 0.0006582 | 0.0015951 | 0.0025417 |
| Hippocampus | 0.0035768 | 0.0028287 | 0.0007592 | 0.0013464 | 0.0019104 |

Figure 9

## Key Research Accomplishments

- $U_3O_8$ does not appear to cause overt cytotoxicity in RBE4 cells.

- $U_3O_8$ failed to change cellular volume in RBE4 cells, an early indicator of cytotoxicity

- $U_3O_8$ increased Hsp90 protein expression in RBE4 cells, but this effect did not appear to be concentration-dependent or time-dependent.

- In vivo studies have been completed and all cohorts have been treated and tissues collected for U analysis

- DU treatments led to no signs of overt toxicity in the rat model. There were no indications for failure to gain weight, no indications of increased infections or malaise, and all animals have survived treatments up to 6 months of exposure

- Brain U concentrations in rats exposed to DU do not appear to differ from those in controls. Both in cortex and hippocampus there appear to be no statistical differences in U brain concentrations between controls and DU implanted rats

- Since ours is the first study to employ whole animal perfusion prior to sacrifice we believe that the experimental paradigm minimizes contamination of brain (and other tissue samples) by blood-borne U.

## Reportable Outcome

Lack A, Erikson KM, Dobson AW, Aschner M. Uranium uptake in rat brain endothelial cells and the possible link to divalent metal transporter-1 (DMT-1). Toxicol Sci 56, 2003 (abstract).

Once all cohorts are analyzed, we plan to publish a comprehensive report.

## Technical Issues

As of August 1, 2004, the PI, Dr. Michael Aschner has assumed a new position at Vanderbilt University Medical Center. Dr. Aschner serves as the Gray E. B. Stahlman

Endowed Professor of Neuroscience in the Department of
Pediatrics. A request has been made to DoD to allow for the
transfer of funds from years 03 and 04 to the PI's new
institution. There is no other PI at the Wake Forest
University Medical Center with expertise in the
neurotoxicology of heavy metals. Furthermore, the PI had
already invested time and effort in the experimental
design, animal treatment, and collection of samples.
Accordingly, if the study is to be brought to fruition it
is best that the current PI, Dr. Michael Aschner complete
them in his new location. We are eagerly awaiting the
transfer of the funds to allow us to complete the studies.

## Conclusions

In vitro methods such as volume measurements and the
expression of Hsp90 in endothelial cells (RBE4) suggest that
these cells can withstand exposure to relatively high U
levels (up to 100 uM) in the absence of signs of overt
toxicity.

In vivo studies in a rat model implanted with DU pellets for
6 months suggests that the concentrations of brain U in these
animals, specifically in the cortex and hippocampus, areas
referred to as non-target and target regions for U are
indistinguishable from controls. Our studies indicate that U
does not cross the BBB at appreciable amounts in rats
implanted with DU for up to 6 months post implantation.

## References

Pellmar, T. C., Fuciarelli, A. F., Ejnik, J. W., Hamilton, M.,
Hogan, J., Strocko, S., Emond, C., Mottaz, H. M., and
Landauer, M. R.  Distribution of uranium in rats implanted
with depleted uranium pellets.  Toxicol. Sci. 49:29-39, 1999a.

Pellmar, T. C., Keyser, D. O., Emery, C., and Hogan, J.B.
Electrophysiological changes in hippocampal slices isolated
from rats embedded with depleted uranium fragments.
Neurotoxicology 20:785-792, 1999b.